Reference: *Biol. Bull.*, **147**: 213–226. (August, 1974)

BUDDING, SEXUAL REPRODUCTION, AND DEGENERATION IN THE COLONIAL ASCIDIAN, *SYMPLEGMA REPTANS*[1]

KEIJI SUGIMOTO AND MITSUAKI NAKAUCHI

Zoological Institute, Faculty of Science, Tokyo Kyoiku University, Bunkyo-ku, Tokyo 112 and Department of Biology, Kochi University, Asakura, Kochi 780, Japan

Ascidians of the subfamily Botryllinae propagate asexually by pallial budding and, in some genera, by vascular budding (Berrill, 1950; Abbott, 1953; Oka and Watanabe, 1957, 1959; Milkman, 1967). Since Bancroft's pioneer work (1903), many developmental problems have been studied in botryllid ascidians, but most of the studies have been made on *Botryllus* or *Botrylloides,* so our knowledge of the budding in other synstyelid ascidians is very limited. Berrill (1940) described the mode of the budding of *Symplegma viride,* and his work is the only earlier study of budding in this genus.

Symplegma reptans is one of the common synstyelid ascidians in Japanese waters, forming thin, flat colonies. This species is easily cultured on glass plates, providing colonies that are easy to handle and to operate upon, and are clearly observable from both sides. In these points *Symplegma* is much like *Botryllus,* but it has additional features which are desirable for some types of study: (1) the zooids of *Symplegma* are "independent," each zooid possessing its own individual atrial opening; (2) the life span of each zooid is much longer than that of *Botryllus;* and (3) *Symplegma* reproduces sexually throughout the year.

Employing this species a series of descriptive and experimental studies have been carried out by the present authors. In this paper we present our observations on the life history of the species, and give special attention to the quantitative and dynamic aspects of budding. This and coming papers will show that *S. reptans* is excellent material for various kinds of study.

Material and Methods

For a taxonomic description of *Symplegma reptans* (Oka) see Tokioka (1949). Studies were carried out at the Usa Marine Biological Station of Kochi University, from March to May, 1969, at a sea water temperature of 15°–21° C, and at the Shimoda Marine Biological Station of the Tokyo Kyoiku University, from May to July, 1972, at a sea water temperature of 18°–24° C.

Colonies of *Symplegma* collected in the field were brought back to the laboratory with their original substrata. Here the growing margin of the colony was detached, and fragments of this cut by a razor blade were fastened by thread to glass plates and allowed to attach and grow. Oozooids were obtained by placing cultured colonies containing mature zooids in a bowl, and collecting the larvae released from them with a pipette. Then the tadpoles were settled at desired positions on a glass plate. Glass plates bearing fragments of colonies or settled larvae were

[1] Contribution no. 234 from the Shimoda Marine Biological Station.

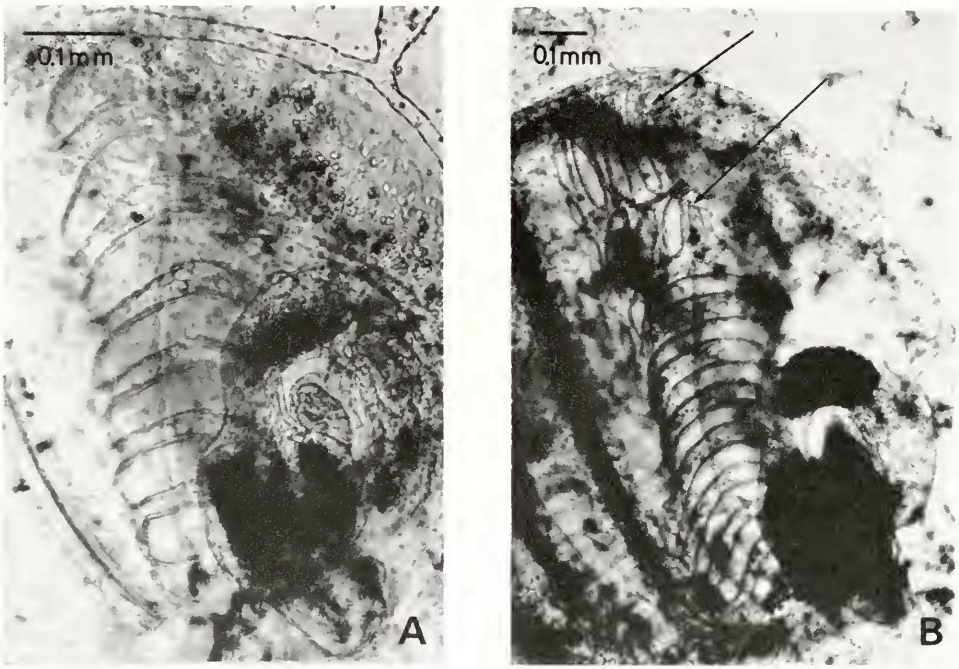

FIGURE. 1. Development of stigmata in the oozooid. (A) 5-day oozooid with 7 pairs of protostigmata, ventral view. (B) 20-day oozooid with 2 rows of stigmata and 8 protostigmata on one side of the branchial basket, ventral view.

cultured in a slidebox set in the bay. Materials were brought back daily to the laboratory, and repeated observations on *particular zooids* were made there.

For histological study the materials were fixed in cold 5% phosphate-buffered glutaraldehyde at pH 7.4 for 3 hours and embedded in Epon. The blocks were sectioned serially at 1 μ and stained with 1% toluidin blue.

OBSERVATIONS

Development, bud formation, and degeneration of the oozooid

In the laboratory most of the larvae are released during the morning hours (10–12 A.M.), and begin to metamorphose within 4–5 hours after release. At a temperature of about 15° C, heart-beat begins 2 days after attachment of larva, and 1–2 days later the apertures perforate and feeding begins. By this stage the body of the zooid is nearly transparent owing to consumption of the yolk with which the newly metamorphosed zooid is filled. The first bud is formed on the oozooid about 10 days after metamorphosis. At a temperature of 20°–23° C, the time schedule is speeded up: it takes 1–2 days after metamorphosis for perforation of the apertures, and only 3 days after metamorphosis the first bud is produced.

The oozoid continues to grow and gives off a series of 4–7 buds during its life span. In about 30 days after metamorphosis the oozooid begins to degenerate. The time of degeneration is little affected by temperature. Colonies grow faster

under warmer conditions, so the size of the colony at the time degeneration of the oozoid occurs varies with the temperature. Degeneration occurs more or less suddenly. It is not preceded by a decrease in the growth rate or by a slowdown of the heart-beat, both of which are observed in the degeneration of blastozooids.

A young functional oozooid has 7–9 protostigmata on each side of the pharynx (Fig. 1A). The anterior 3–4 protostigmata are subdivided successively from anterior to posterior during the growth of the oozooid (Fig. 1B. By the time degeneration occurs the subdivision of the anterior 3–4 pairs is completed, but the posterior 3–5 ones remain undivided. The oozooid thus has at least 3 pairs of protostigmata throughout its life.

The blastozooids produced by successive buddings of an oozooid spread out radially and surround the oozooid. These zooids in turn produce buds, so at the time of degeneration the oozooid is situated at the center of the colony composed of 70–80 zooids at various stages of development.

No gonad is formed in the oozooid before it degenerates. Even in oozooids which were surrounded by blastozooids with visible gonads no sign of gonad formation was observed.

Development and bud formation of the blastozooid

As stated above, the first bud, or first blastozooid, of an oozooid is formed about 10 days after metamorphosis at about 15° C. The bud arises from a definite site on the antero-ventral wall of the right atrial chamber. This site is very close to the base of a test vessel (Fig. 2A). The second and the subsequent buds are also formed in the same region. The epidermis and atrial epithelium of this region thicken and form a double-layered hemispheric vesicle about 50 μ in diameter at this stage. With further development the bud becomes nearly spherical. The inner layer of the bud then detaches from the parental atrial epithelium whereas the outer layer remains continuous with the parent wall by a broad stalk through which the blood flows (Fig. 2B). Within 24 hours after the formation of a hemisphere vesicle the stalk is broken. However, a new test vessel arises from the anterior wall of the bud (Fig. 2C); this fuses with a test vessel of the parent, so the developing bud is again connected with the parental circulatory system.

The developing bud, or blastozooid, forms its own first bud at a very early stage of development, when it is only 3–4 days old (17–20° C) and is not yet functional (Fig. 2D). At this stage the developing zooid is about 0.3 mm in length and 0.26 mm in width. The heart becomes functional when the developing zooid is 6–7 days old (17°–20° C) and has reached an average size of 0.39 × 0.29 mm (Fig. 2E). One day after the heart begins to beat the apertures become perforated (Fig. 2F). After this the zooids feed actively and grow quickly (Fig .3).

Each developing zooid gives rise to a series of 3–6 buds during the period of development and growth (Fig. 4). The budding phase of a blastozooid comes to an end at about the time a pair of gonads appear clearly. At this stage the zooid measures about 1.7 mm in length, and 1.3 mm in width. The oocyte of this stage is about 35 μ in diameter. As a rule, no bud formation occurs after this stage, and the gonad develops quickly. In other words, the budding, or growing phase gives place to the sexual reproduction phase. It takes, on an average,

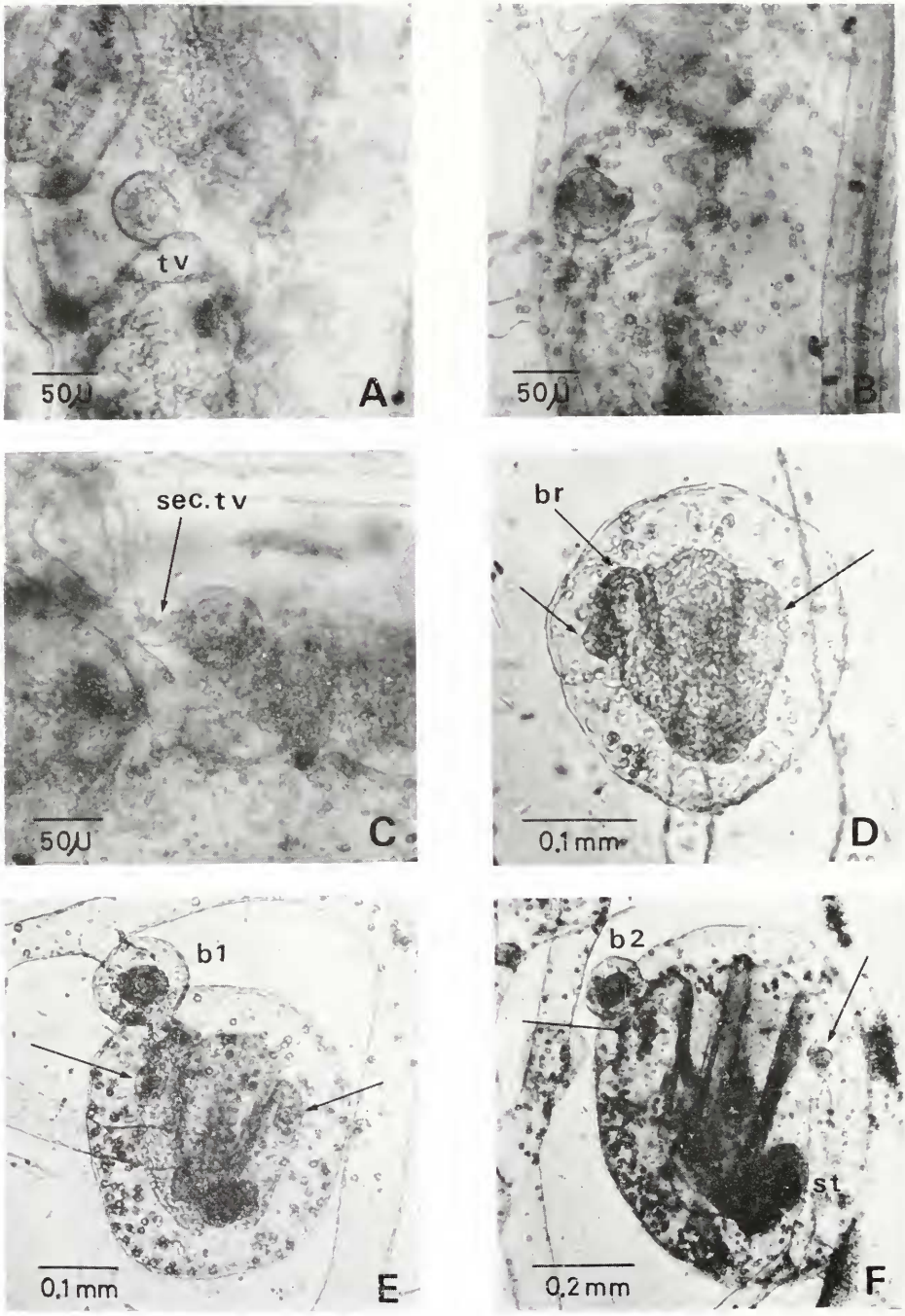

FIGURE 2. Development of a bud arising from an oozooid. (A) Appearance of bud vesicle. (B) Bud vesicle with a broad test vessel. (C) 1-day bud with a secondary test vessel. (D)

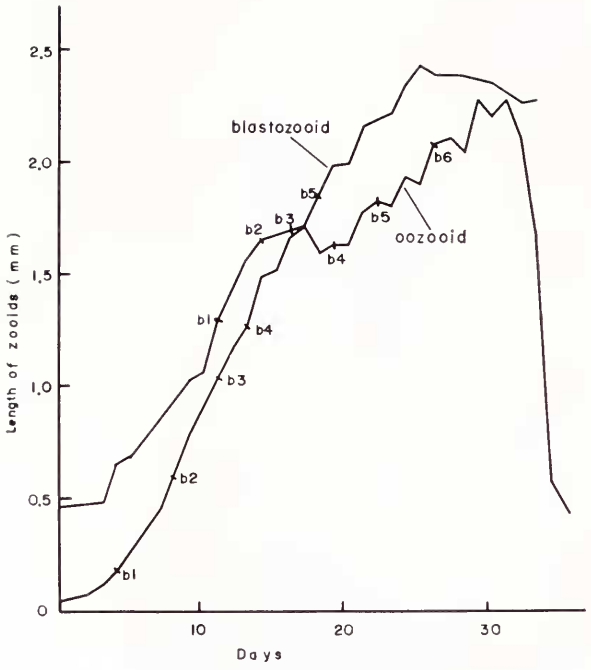

FIGURE 3. Growth of an average oozooid and an average blastozooid. Abbreviations are the same as Figure 2.

14.6 days (18°–24° C) to reach this stage after the "birth" of a blastozooid as a bud rudiment (Figs. 5, 6).

The buds formed on the wall of a developing blastozooid develop in the same manner as the buds formed on an oozooid. No significant differences have been found between the time schedule of development of buds derived from an oozooid and that of buds derived from a blastozooid. Blastozooids derived from blastozooids also produce a succession of 3–6 buds during their budding phase. They stop bud formation on an average of 12.8 days after their birth, and enter the sexual phase.

Buds and young zooids spread out to the periphery of the colony, and older zooids remain in the central region. A large colony thus consists of two kinds of zooids with respect to reproductive activity; zooids in sexual phase which occupy the central region of the colony and zooids in the asexual phase occupying the periphery.

The intervals of the successive buddings in each oozooid and in each blastozooid are summarized in Table I. At about 17° C, the first budding of the oozooid takes place later than at about 20° C. However, the intervals between successive

3-day bud in which the first bud of the next generation is being formed. (E) 6-day bud in which weak heart-beat is seen. (F) 8-day blastozooid which has begun feeding. Arrows indicate gonadial rudiment; b1 (b2), first (second) bud; br, thickening of peribranchial wall as a bud rudiment; sec. tv, secondary test vessel; st, stomach; tv, test vessel.

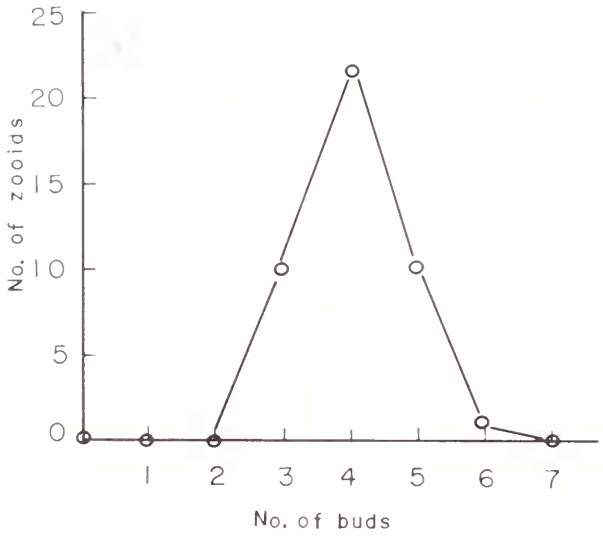

FIGURE 4. Variation in the number of buds produced per blastozooid.

buddings in oozooids at 20° C is almost same as that in blastozooids at 20° C; in both, budding occurs every 2–3 days (Fig. 7). The 6th and 7th buddings tend to occur after a longer interval than earlier ones.

The blastozooids surrounding an oozooid sometimes close their budding phase before the oozooid begins to degenerate. The buds produced by old oozooids and aged blastozooids tend to become abortive.

Sexual reproduction and degeneration of the blastozooid

The oozooid reproduces only asexually during its life span. The blastozooid reproduces asexually until the gonads have developed to the stage where a pair of

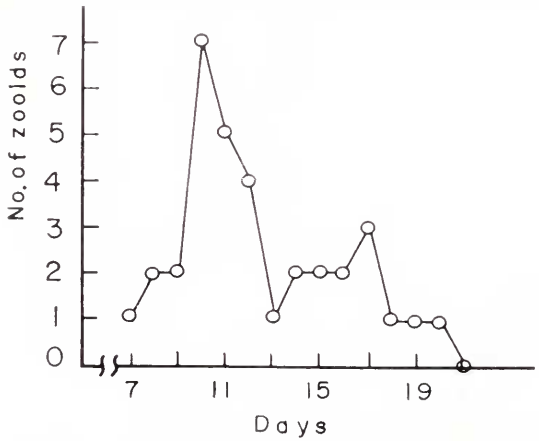

FIGURE 5. Age in days of blastozooids at the time of formation of the last bud.

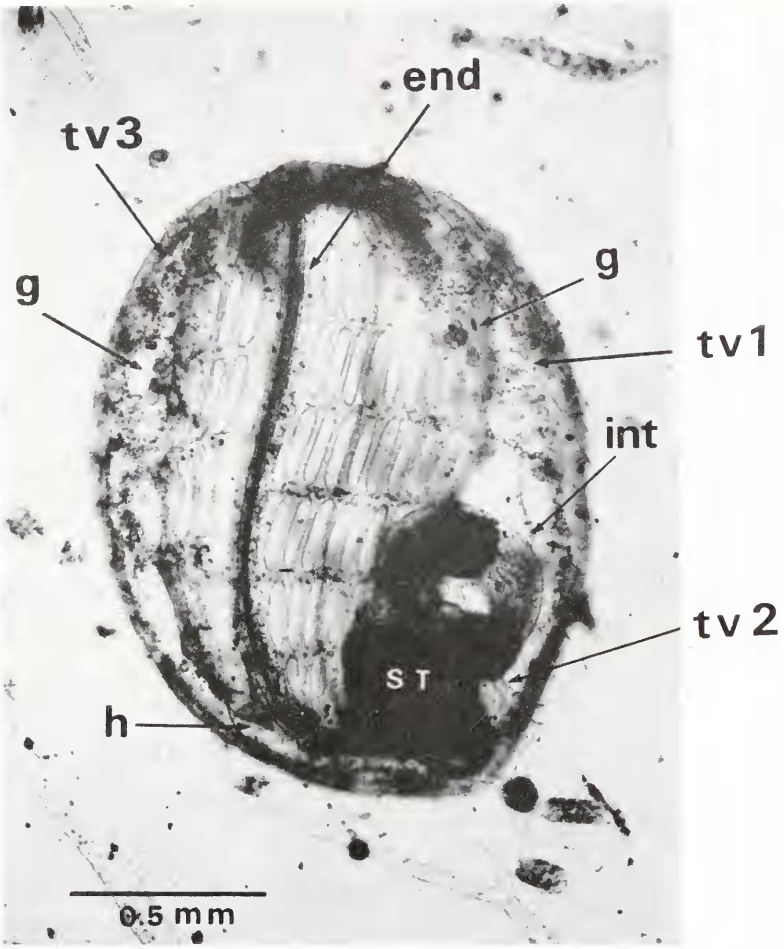

FIGURE 6. The 13-day blastozooid which has just stopped bud formation; end, endostyle; g, gonad; h, heart; int, intestine; st, stomach; tv(1–3), test vessel.

TABLE I

Average intervals between two successive buddings in each zooid (15°–21°C)

	Oozooid	Blastozooid
Metamorphosis to 1st budding	10.8 (days)	
Blastozooid "birth" to 1st budding		4.1 (days)
1st–2nd budding	2.3	2.2
2nd–3rd budding	2.5	2.2
3rd–4th budding	4.0	2.5
4th–5th budding	2.8	3.0
5th–6th budding	4.0	4.0
6th–7th budding	8.0	

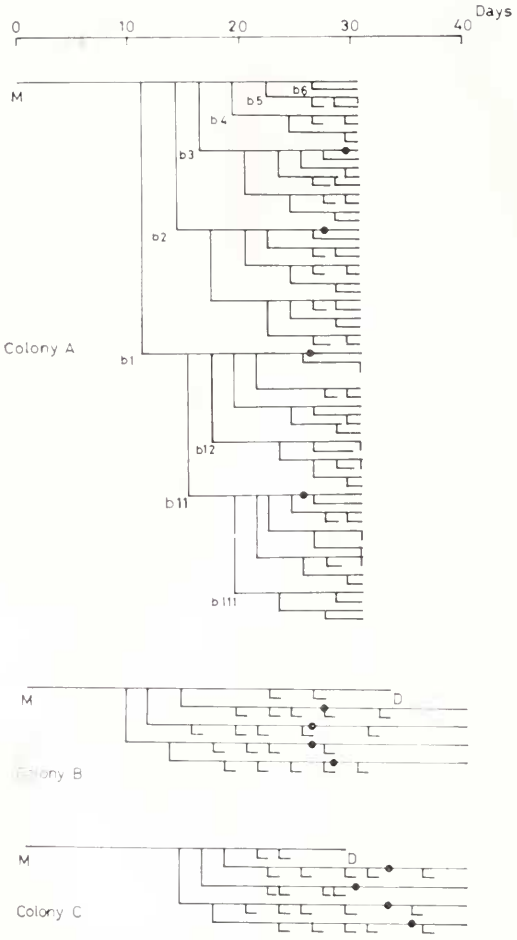

FIGURE 7. Genealogies of three colonies originating from oozooids. Nomenclature of the zooids is taken from Berrill (1940). The end of lines indicates the end of observation of the zooids; D, degeneration of the zooid; M, metamorphosis; solid circle, appearance of gonads.

testicular follicles and an ovarian follicle are clearly observable under a microscope in living materials.

The sexual phase starts with this stage, but the gonad rudiment appears much earlier. It appears as a mass of lymphocytic cells in the genital tracts around the time when the first bud is formed in the developing blastozooid (Fig. 8A). In living materials so many blood cells are found in the genital tracts that it is impossible to identify the gonadial rudiment, but in fixed material identification is easier. The gonadial rudiment grows as the zooid grows (Figs. 8B–8D). As shown in Figures 9A–9D, the eldest oocyte enters to quick-growing phase about 20 days (18–24 C) after the birth of the blastozooid. In about 5 days the growing oocyte reaches its maximum size, 220 μ in diameter.

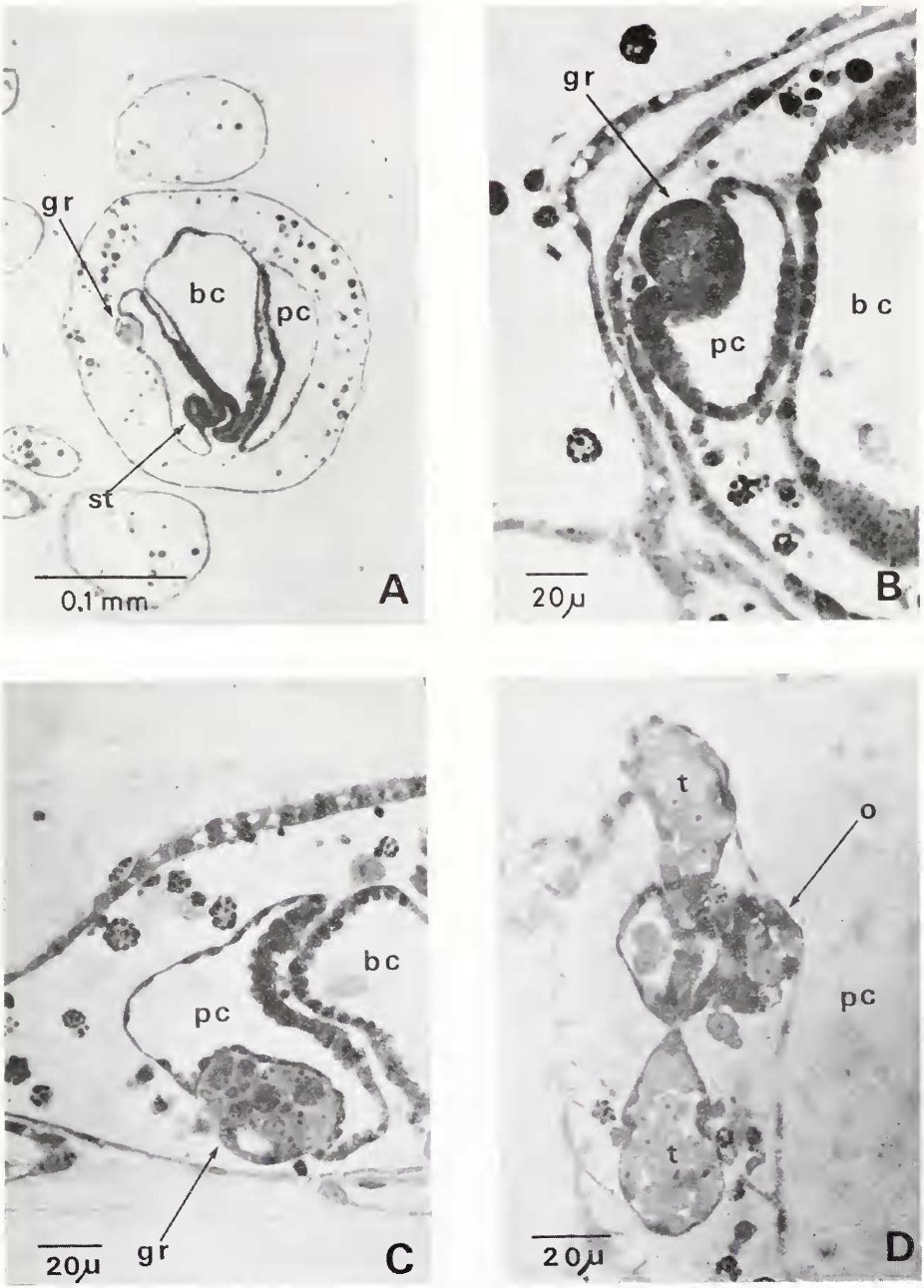

FIGURE 8. Formation of the gonadial rudiment by an aggregation of lymphocytic cells; (A) frontal section of a 4-day blastozooid, showing the position of the gonadial rudiment; (B) gonadial rudiment of a 4-day blastozooid; (C) longitudinal section of a 5-day blastozooid; (D) gonad of an 11-day blastozooid; bc, branchial cavity; gr, gonadial rudiment; o, ovary; pc, peribranchial cavity; st, stomach; t, testis.

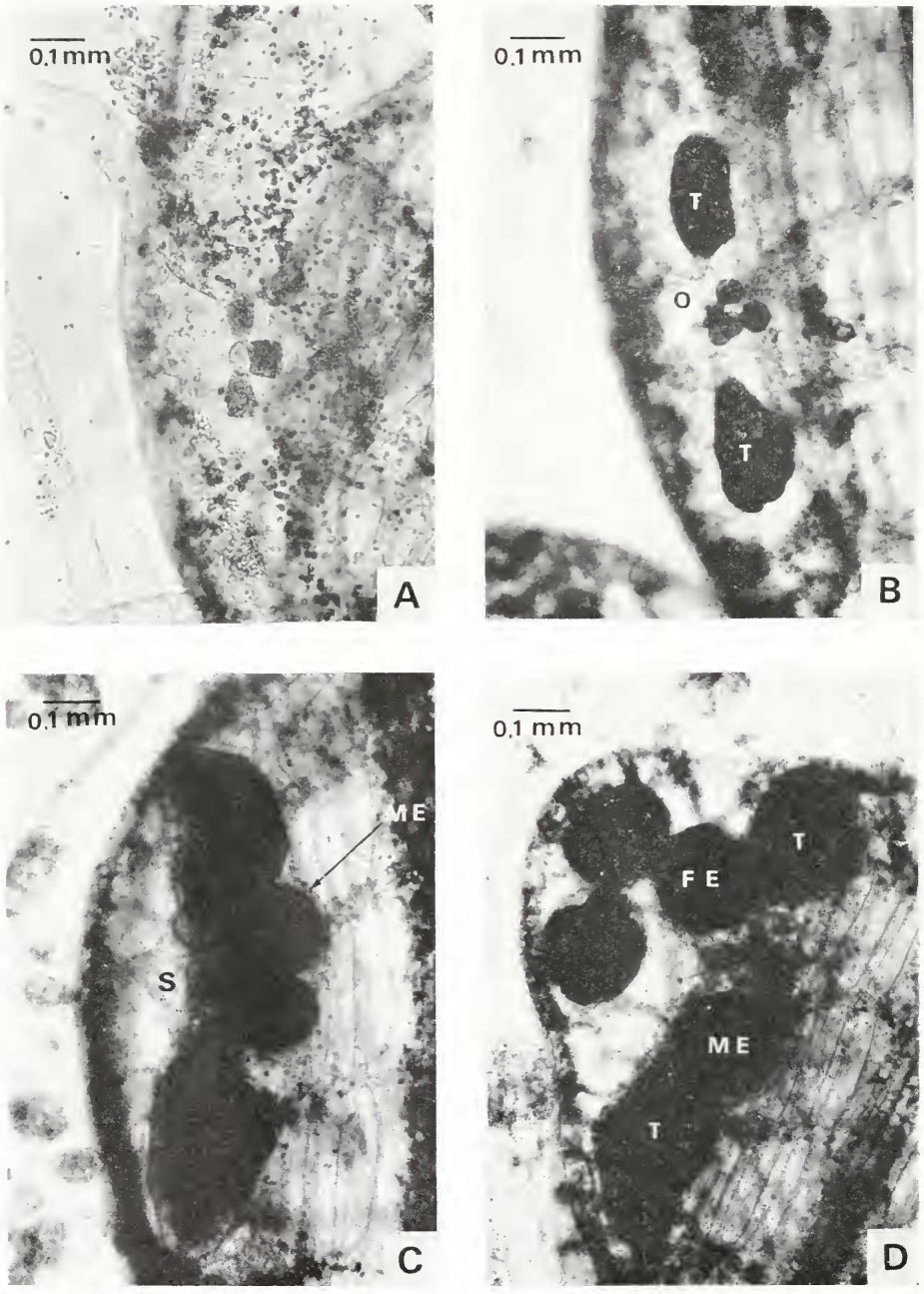

FIGURE 9. Development of the gonad; (A) 11-day zooid; (B) 15-day zooid; (C) 24-day zooid; (D) 28-day zooid; FE, fertilized egg; ME, mature egg, O, ovary; S, brood sac; T, testis.

With enlargement of the oocytes, the shape of the zooid changes from ellipsoidal to triangular. In the anterior region on both sides of the body the epidermal and peribranchial wall protrude laterally, and the anterior part of each peribranchial cavity expands laterally. A brood sac derived from the ovary protrudes into each expanded cavity (Fig. 10A). After the eggs have reached maximum size they are ovulated in the brood sac where fertilization occurs and development of the tadpole larvae takes place.

In the early stage of gonad development, 6–7 oocytes are seen in the ovary. Before these oocytes reach their maximum size, new young oocytes appear. Further oocytes also appear as the enlarged ova are ovulated. So, in the aged zooid, 5–7 oocytes of various stages and about 3 ova are seen in the ovary. Sometimes, the larva developing in a brood sac moves the tail actively. At a temperature of 18°–24° C, it takes 30 days from the birth of the blastozooid, and 5 days from fertilization, to the liberation of the first larva.

Some of the blastozooids have been observed to degenerate in 60–70 days after their birth. In these zooids the internal organs except for the heart begin to disintegrate, and the zooids become transparent (Fig. 10B). These zooids contain a few larvae or ripening eggs, but before degeneration is complete all the larvae are released. From the number of cases oberved we cannot say that the life span of the blastozooid is limited to 60–70 days, but it is clear that blastozooids live for 60 days or more, and that at least some of them degenerate before the death or degeneration of the whole "colony." Time schedules of development for an average oozooid and an average blastozooid are shown in Figure 11.

Discussion

While the mode of budding of *S. reptans* is shown to be fundamentally identical with that of *S. viride* as reported by Berrill (1940), several new facts are revealed by the present study. The time schedule of budding and degeneration of zooids were previously unknown, and the development of the buds arising from the oozooid was "described only partially" by Berrill (1940). Repeated observations on particular oozooids in the present study reveals that the oozooid makes its first

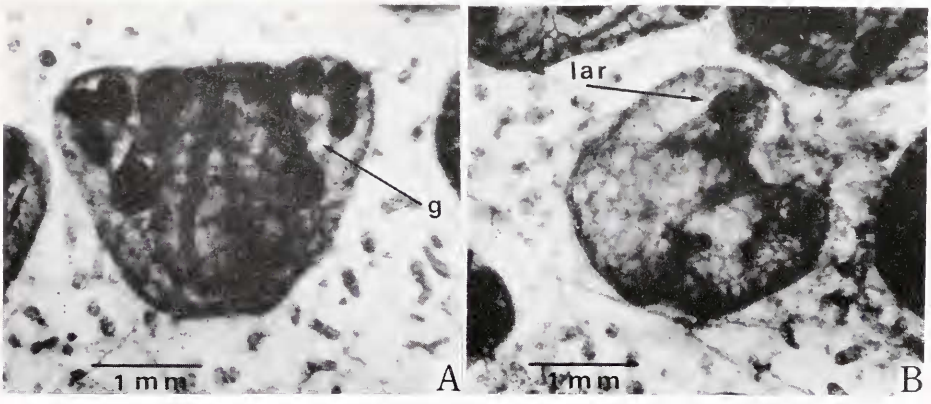

Figure 10. (A) Fully mature 35-day blastozooid with larvae; (B) Degenerating 65-day blastozooid in which all organs but the heart have disintegrated; g, gonad; lar, larva.

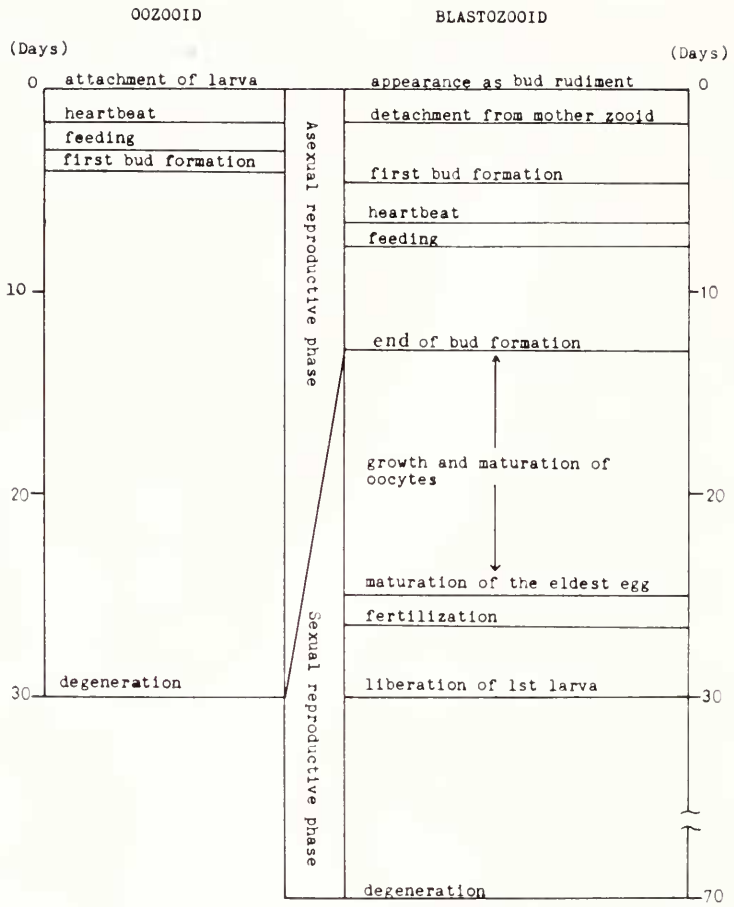

FIGURE 11. Time schedules of development in average oozooids and blastozooids at about 20° C.

bud about 10 days (17°–20° C) after metamorphosis and thenceforth it continues to produce buds at intervals of 2–8 days until it degenerates. The oozooid produces 4–7 buds during its life span of about 30 days.

It is also shown that in oozooids neither complete subdivision of the protostigmata nor gonad formation takes place before degeneration begins, so the oozooid is distinguishable from the blastozooids in the same colony by these morphological characters. It is of interest that, likewise, no gonad is formed in the oozooid of the synstyelid *Metandrocarpa taylori* (Watanabe, 1970) or in that of the pelagic tunicate *Doliolum* (Berrill, 1950).

As to the number of the buds produced by each blastozooid, Berrill (1940) states that in *S. viride*, "As far as can be determined, this (fourth bud) represents the last of the bud series." The present study shows that in *S. reptans* the fourth bud is not always the last bud, and that the number of buds produced by a developing blastozooid varies from 3 to 6.

In *S. reptans* the budding phase of a blastozooid comes to an end about the time when a pair of gonads becomes clearly visible, and as a rule no budding takes place in mature zooids. However, the rudiment of gonad is shown to be formed early in the asexual phase, so here the sexual phase is not so clearly segregated from the asexual one as in *Hydra* (Loomis and Lenhoff, 1956; Loomis, 1959) or in the hydroid *Podocoryne* (Braverman, 1962).

At an early stage of gonad formation the lymphocytes were found to aggregate in the genital tracts. The same phenomenon was seen in *Distomas variolosus* (Newberry, 1968) and in *Botryllus primigenus* (Mukai and Watanabe, 1972). These observations suggest that lymphocytes are usually involved in gonad formation in synstyelid ascidians. One of the most important problems is whether or not these lymphocytes are on the so-called germ cell line. Growing oocytes at various stages have been observed in the common blood system of *Botryllus* (Berrill, 1941; Izzard, 1968; Mukai and Watanabe, 1972), but such oocytes are not found in the test vessels of *S. reptans*. As far as the microscopical sections show, germ cells seem to arise directly from the mass of the lymphocytes. The lymphocytes of ascidians are also known to play an important role in the budding of *Botryllus* (Oka and Watanabe, 1957; Milkman, 1967), *Botrylloides* (Oka and Watanabe, 1959), and *Perophora* (Freeman, 1964). These facts call for further studies on the developmental capacities of ascidian lymphocytes. It would also be desirable to trace the origin of the lymphocytes found in the genital tracts, to see whether the germ cells of ascidians are determined in an early stage of development as in mammals and insects.

As to the life spans of the zooids of *Symplegma,* Berrill (1940) states, "unlike *Botryllus,* the constituent zooids of a colony of *Symplegma* persist while the colony lives and are not brief transients." Our observations show that oozooids of *S. reptans* degenerate abut 30 days after metamorphosis, while the blastozooids of the same colony remain healthy. The oozooid of *Aplidium californicum* also degenerates in 23–32 days after metamorphosis, whereas all the blastozooids of the same colony are very active (Nakauchi, unpublished). The factors governing the degeneration of particular individuals in a *Symplegma* colony are being investigated.

The authors wish to express their thanks to Professor H. Watanabe of Tokyo Kyoiku University for his valuable advice and encouragement during this study, and to Professor D. P. Abbott of the Hopkins Marine Station of Stanford University for his critical reading of the manuscript. The authors are also grateful to the staff of the Usa Marine Biological Station and the Shimoda Marine Biological Station for their kindness in providing many conveniences throughout the course of the study.

Summary

The life history of *Symplegma reptans* was studied by repeated observations on particular living animals and by observations on sectioned materials.

The oozooid produces its first bud about 10 days (17°–20° C) after metamorphosis, and produces a series of 4–7 buds by the time the oozooid begins to degenerate. The degeneration occurs about 30 days after metamorphosis. No

gonad is formed in the oozooid even at the time when gonads are seen in the blastozooids of the same colony. Of the 7–9 protostigmata present in the newly metamorphosed oozooid, the anterior 3–4 are subdivided by the time of degeneration, but the posterior 3–5 remain undivided throughout the life of the oozooid.

A blastozooid makes its own first bud 3–4 days (17°–20° C) after its first appearance, when it is still scarcely more than "bud" itself. The growing blastozooid makes a succession of 3–6 buds at intervals of 2–4 days. Bud formation by a blastozooid come to an end about the time its gonad become macroscopically visible. Blastozooids live for 60 days or more, but at least some of them degenerate before the death or degeneration of the colony.

Microscopical sections suggest that the germ cells are formed directly from lymphocytes.

LITERATURE CITED

Abbott, D. P., 1953. Asexual reproduction in the colonial ascidian *Metandrocarpa taylori* Huntsman. *Univ. Calif. Publ. Zool.*, **61**: 1–78.

Bancroft, F. W., 1903. Variation and fusion of colonies in compound ascidians. *Proc. Calif. Acad. Sci.* (Sci. 3), **3**: 137–186.

Berrill, N. J., 1940. The development of a colonial organism: *Symplegma viride*. *Biol. Bull.*, **79**: 272–281.

Berrill, N. J., 1941. The development of the bud in *Botryllus*. *Biol. Bull.*, **80**: 169–184.

Berrill, N. J., 1950. *The Tunicata with an Account of the British Species*. Ray Society, London. 354 pp.

Braverman, M. H., 1962. Studies in hydroid differentiation. I. *Podocoryne carnea*, culture methods and dioxide induced sexuality. *Exp. Cell Res.*, **26**: 301–306.

Freeman, G., 1964. The role of the blood cells in the process of asexual reproduction in the tunicate *Perophora viridis*. *J. Exp. Zool.*, **156**: 157–183.

Izzard, C. S., 1968. Migration of germ cells through successive generations of pallial buds in *Botryllus schlosseri*. *Biol. Bull.*, **135**: 424.

Loomis, W. F., 1959. Feedback control of growth and differentiation by carbon dioxide tension and related metabolic variables. Pages 253–293 in D. Rudnick, Ed., *Cell Organisms and Milieu*. The Ronald Press Co., New York. 326 pp.

Loomis, W. F., and H. M. Lenhoff, 1956. Growth and sexual differentiation of *Hydra* in mass culture. *J. Exp. Zool.*, **132**: 555–573.

Milkman, R., 1967. Genetic and developmental studies on *Botryllus schlosseri*. *Biol. Bull.*, **132**: 229–243.

Mukai, H., and H. Watanabe, 1972. Gametogenesis in *Botryllus primigenus*. *Zool. Mag.* (*Tokyo*) (in Japanese), **81**: 268.

Newberry, A. T., 1968. The gonads and sexual cycle of the polystyelid ascidian *Distomus variolosus* Gaertner. *J. Morphol.*, **126**: 123–162.

Oka, H., and H. Watanabe, 1957. Vascular budding, a new type of budding in *Botryllus*. *Biol. Bull.*, **112**: 225–240.

Oka, H., and H. Watanabe, 1959. Vascular budding in *Botrylloides*. *Biol. Bull.*, **177**: 340–346.

Tokioka, T., 1949. Contribution to Japanese ascidian fauna II. Notes on some ascidians collected chiefly along the coast of Kii peninsula. *Publ. Seto Mar. Biol. Lab.*, **1**: 39–64.

Watanabe, H., 1970. Asexual reproduction in compound ascidians (I). *Zool. Mag.* (*Tokyo*) (in Japanese), **79**: 131–143.